Insight into the interfacial self-assembly and structural changes of hydrophobins
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CHAPTER 4

Probing the self-assembly on a hydrophobic surface and structural changes of hydrophobin SC3 by mass spectrometry

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Submitted for publication
Abstract

Hydrophobin SC3 undergoes large conformational changes during the interfacial self-assembly process. SC3 bound to a Teflon surface can be in the α-helical state or β-sheet state, whereas the protein at the air/water interface is uniquely in the β-sheet state. The structure of SC3 in these various states was probed by mass spectrometry. Performic acid-oxidized SC3 (PFA-SC3) could be specifically digested by endoproteinase Asp-N, and the peptides with high hydrophobic-surface binding affinities could be identified by matrix-assisted laser-desorption/ionization – time-of-flight (MALDI-TOF) mass spectrometry. It was found that a portion of the predicted 2nd loop (C39-S72) was not only binding to the surface, but was also involved in the formation of α-helical-state structure. This conclusion was fully supported by tandem electrospray mass spectrometry (ESI-MS/MS) in combination with amide hydrogen/deuterium exchange experiments performed on soluble-state SC3, Teflon-bound PFA-SC3 either in the α-helical or β-sheet state, and Ca\textsuperscript{2+}-associated PFA-SC3 in an α-helical-like state. Upon formation of the α-helical or β-sheet state on a Teflon surface, the first half of the predicted 2nd loop exhibited very slow deuterium exchange, compared to the situation in the soluble state, indicating that, upon surface binding, a dramatic disorder→order change takes place in this region of the molecule. This conclusion was confirmed by the fact that a chemically synthesized peptide, which corresponds to this part of the molecule, gained α-helical-like structure upon binding to a Teflon surface.
**Introduction**

Hydrophobins are a family of small fungal proteins that self-assemble at hydrophilic/hydrophobic interfaces, such as water/air, water/oil, and water/solid-hydrophobic-surfaces, into an amphipathic membrane that forms a robust monolayer composed of rodlet structures (Wösten et al., 1993; Wösten et al., 1994a,b,c; van Wetter et al., 2000a). The rodlets mimic amyloid fibrils, in as much as both bind the dyes Thioflavine T and Congo red (de Vocht et al., 2000; Mackay et al., 2001; Butko et al., 2001). Therefore, hydrophobins have been suggested to represent biologically functional amyloid proteins, that facilitate the emergence of fungal aerial hyphae or spores from the medium or their aerial dispersal (Wösten et al., 1994a; 1999). Due to their remarkable surface activity, hydrophobins have potential industrial applications, such as surface patterning with nanometer accuracy, immobilisation of antibodies in a biosensor, and drug delivery using oil vesicles stabilised with a hydrophobin.

Hydrophobins have diverse amino acids sequences, but eight conserved cysteine residues. Based on their hydropathy patterns, the number of amino acids between the third and fourth cysteine residue and their solubility characteristics, hydrophobins are classified into two classes, class I and class II hydrophobins (Wessels, 1994,1997; Wösten et al.,1997). Hydrophobin SC3, secreted by *Schizophyllum commune*, is a class I hydrophobin whose eight cysteine residues are assumed to form four disulfide bridges, which separate the molecule into four loops, with the second one being the largest (32 amino acids). In addition, SC3 has a 29-amino acid N-terminal sequence preceding the first cysteine residue, where 16-22 O-linked mannose residues are believed to be attached to threonine residues. Deglycosylation of SC3 does not affect the self-assembly, except that the amphipathic layer formed on a Teflon surface has a higher water contact angle than a Teflon surface modified with the glycosylated protein, indicating that the mannose residues are present on the hydrophilic side of the layer (de Vocht et al., 1998). The disulfide bridges in SC3 are not directly involved in self-assembly, but function to stabilize the molecules in the soluble state. Once encountering a Teflon surface, iodoacetic acid-modified SC3 (IAA-SC3), with the eight cysteine residues carboxymethylated and, therefore, carrying eight negative charges, could refold from a random coil state in solution into an α-helical-like structure similar to native SC3 on a Teflon surface (de Vocht et al., 2000).

There are at least three different conformational states known for SC3, the soluble state, the α-helical state and the β-sheet state (including non-rodlet β-sheet I and rodlet β-sheet II state). The α-helical state forms spontaneously once soluble-state SC3 comes in contact with a hydrophobic surface such as Teflon in water. It can be converted to the end state, β-sheet II state by heating.
up the sample on Teflon in the presence of detergent. The β-sheet state of SC3 in the present study only refers to β-sheet II state. The increase in β-sheet structure upon SC3 self-assembly on a Teflon surface leads to the rodlets. In contrast to the situation on a Teflon surface, the β-sheet II state forms spontaneously once soluble-state SC3 encounters an air/water interface; this happens via a short-lived α-helical state and β-sheet I state (de Vocht et al., 1998; de Vocht et al., 2002; Wang et al., 2002). SC3 in the soluble-state is mainly dimeric with a lower amount of secondary structural elements than the other two states (Wang et al., unpublished).

The structure/function relationships of hydrophobin SC3 are poorly understood. Recently, a low-resolution structure of self-assembled fibrils of the class II hydrophobin HFBII from Trichoderma reesei was presented which was derived from small and wide-angle X-ray scattering. The protein was characterized as a tetramer in aqueous solution, whereas a monoclinic crystalline structure was found in the undried fibrils and suggested to be formed by stacking the repeat tetramers (Torkkeli et al., 2002). The soluble-state structure of another class I hydrophobin, EAS from the ascomycete Neurospora crassa, has been studied by NMR. It was found that EAS is largely unstructured in solution, except for a small core region composed of three anti-parallel β-strands, which are probably stabilized by the four disulfide bridges (Mackay et al., 2001).

The folding in solution and at a water/hexane interface of hydrophobin SC3, lacking the N-terminal glycosylated part, has been recently subjected to molecular dynamics simulations (MD). Similar to EAS, this N-terminal truncated SC3 was found to be a largely unstructured globular protein in solution, which may undergoes a rapid disorder-to-order folding process, leading to an elongated planar structure with extensive β-sheet secondary elements, at a water/hexane interface (Zangi et al., 2002). The formation of β-sheets at the interface was a dynamic process with additional β-sheet strands added from other parts of the molecule to an original two-stranded β-sheet element, which is located at the 4th predicted loop.

Secondary structure predictions suggest that the region T52-S63 in the predicted 2nd loop has a high tendency to form an amphipathic helix. The hydrophobic face of this helix might be responsible for the binding of the SC3 to a solid hydrophobic surface (de Vocht et al., 1998). This possibility has been experimentally confirmed in the present study, using Asp-N fragmentation of the protein in combination with matrix-assisted-laser-desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Structural details of various states of SC3 were also elucidated by hydrogen/deuterium exchange (H/D exchange) in combination with electrospray-ionization mass spectrometry (ESI-MS).
Results

Endoproteinase Asp-N fragmentation of performic acid-oxidized SC3 (PFA-SC3) and MALDI-TOF mass spectrometry analysis

1. PFA-SC3 could be specifically fragmented by endoproteinase Asp-N at cysteic acids

Performic acid is able to open a disulfide bond in a protein and oxidize the sulfur group to a stable oxidation state with a yield of about 90%. Methionine and tyrosine in a protein can also be modified by performic acid, while oxidation of hydroxy-amino acids requires more drastic conditions (Sun et al., 1988; Chowdhury et al., 1995). Since SC3 lacks methionine and aromatic residues, performic acid oxidation should specifically convert the eight cysteines into cysteic acids, which might be utilized as endoproteinase Asp-N cleavage sites. The procedure is described in materials and methods. PFA-SC3 was found to be totally unstructured without the tendency to assemble or aggregate in solution (see next section). On the basis of SDS-PAGE (data not shown), the subsequent Asp-N digestion was complete after overnight incubation at 37°C. The peptides generated were assigned to different fragments of SC3 on the basis of MALDI-TOF analysis of their molecular weights, taking into account the mass increase caused by sodium adducts (Fig. 1, Table1). There was only one peptide (labeled with asterisk in Fig. 2A) that could not be assigned to any part of the molecule. Most of the SC3 sequence was covered by the assigned peptides, except for the N-terminal glycosylated domain, which was probably missing due to poor ionization of the fragment. The highest peak in Fig. 2A,B has a mass of 656.2 Da, which might be a mixture of matrix by-product and peptide C86-V91. Since there is no way to distinguish these two species in linear mode detection, it will not be treated further in this study.

Fig. 1 Amino acid sequence of performic acid oxidized SC3 (PFA-SC3). Arrows indicate the peptides generated by endoproteinase Asp-N digestion. The asterisks indicate the cysteines oxidized upon PFA treatment.
Fig. 2 MALDI-TOF analysis on the Asp-N digestion mixture of PFA-SC3

A. Digestion mixture directly analyzed using linear mode MS. The peak labeled with asterisk could not be assigned to PFA-SC3.
B. Digestion mixture directly analyzed using reflectron mode MS.
C. Analysis using reflectron mode after the sample was concentrated and desalted by using ZipTip\textsubscript{C18}.

Both linear and reflectron detection modes were used in the MALDI-TOF analysis, with the former showing better spectra (Fig. 2A,B). However, the accurate monoisotopic mass determination was mainly based on reflectron mode spectra using external calibration. The use of a ZipTip\textsubscript{C18} to desalt and concentrate the sample not only improved the quality of the spectra, but also resulted in the identification of some peptides which were barely detectable in the samples without ZipTip\textsubscript{C18} purification; examples are the peptides D64-S72 (951.4 Da) and C73-G85 (1238 Da) (Fig. 2C). On the other hand, ZipTip extraction also caused the loss of some peptides which showed up in the samples without purification, such as peptide C106-L112. Therefore, sample preparation with and without ZipTip extraction, and MS detection in both linear and reflectron mode were needed to determine the peptide composition of the digestion mixture. In the following fishing experiment, only linear detection mode was used because of the higher recovery of the peptides and the higher peak intensities from samples applied to a Teflon target.
Mass spectrometry analysis of SC3

2. “Fishing” of bound peptides by using a Teflon target

The use of a Teflon instead of a stainless-steel target in MALDI-TOF analysis has been reported to improve the quality of spectra and to increase the number of detectable peptides (Yuan et al., 2002). The advantage of this method is that the peptides bound to the target can be quickly desalted and concentrated by in situ washing steps. In our experiment, after Asp-N digestion of PFA-SC3, the digestion mixture was loaded onto both Teflon and stainless-steel targets. Neither the quality of the spectrum nor the number of assigned peptides was improved when a Teflon target was used. However, the detection of peptides with a high affinity for Teflon proved possible. The setup of the experiment is described in materials and methods. Two of Asp-N digested samples were loaded onto Teflon targets. The sample preparation without a washing step yielded a spectrum similar to that using a steel target, whereas the washed sample resulted in one major peak, a peptide with a molecular weight of 2623 (Fig. 3A,B). This peptide, C39-S63, corresponds to the first half of predicted 2nd loop of SC3, indicating that this part of SC3 binds strongly to a hydrophobic surface. The second half of predicted 2nd loop (D64-S72) might also bind to the surface to some extent, as seen by the presence of a peak at 951 Da in Fig. 3B. This was confirmed by the experiment using colloidal Teflon (see next section).

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<th>Measured m/z</th>
<th>Number of clustered sodium</th>
<th>Theoretical m/z</th>
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</thead>
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<td>2622.7</td>
<td>3</td>
<td>2622.3</td>
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<tr>
<td>2 D64-S72</td>
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<tr>
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<tr>
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<td>1469.7</td>
<td>1514.4</td>
<td>2</td>
<td>1514.7</td>
</tr>
<tr>
<td>5 C’106-L112</td>
<td>820.4</td>
<td>865.3</td>
<td>2</td>
<td>865.4</td>
</tr>
</tbody>
</table>

The asterisks indicate the oxidized cysteines upon PFA treatment. All the peptide masses listed are monoisotopic masses.

3. “Fishing” of bound peptides by using colloidal Teflon, and determination of the corresponding secondary structures by CD

The Asp-N-generated peptides with a high hydrophobic surface binding affinity could also be
separated from the others by using colloidal Teflon. This approach allowed CD measurements and MS analysis to be done on the same material. The experimental details are described in the materials and methods section. Although Asp-N-digested PFA-SC3 in solution, after removing Teflon-bound species, showed a CD spectrum with ellipticity minimum at about 200nm, typical of unstructured proteins/peptides (thick solid line, Fig. 4A), the CD spectrum of the peptides still bound to the Teflon beads showed a typical $\alpha$-helical-state (thin solid line, Fig. 4A). Heating up this Teflon sample to 65°C did not alter the CD spectrum, but the subsequent addition of detergent removed some of the peptides from the Teflon surface and yielded a spectrum of the peptide in solution more typical of an unstructured peptide (dotted line, Fig. 4A).

The supernatant and the centrifuged Teflon beads (in the absence of detergent) were freeze-dried, treated with TFA, and subjected to MALDI-TOF analysis as described. The most abundant peptide in the supernatant was C93-G105, corresponding to the predicted 4th loop. This indicates that this region of the molecule is obviously not preferentially interacting with the hydrophobic surface (Fig. 4B). The most abundant peptides in the resuspended Teflon fraction

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**Fig. 3** Fishing of PFA-SC3 peptides with high binding affinity to a Teflon target
A. Linear mode MALDI-TOF analysis of the digestion mixture on a Teflon target without washing steps. The peak labeled with asterisk could not be assigned to PFA-SC3.
B. Linear mode MALDI-TOF analysis of the digestion mixture on a Teflon target which was washed with water and then overlaid with matrix compound.
were C39-S63 and D64-S72, which correspond to the entire predicted 2nd loop of SC3 (Fig. 4C). These experiments not only confirm the data obtained with the Teflon target, but also suggest that the predicted 2nd loop forms an α-helical-state structure upon binding to a hydrophobic surface.

**Fig. 4** Fishing of PFA-SC3 peptides with high/low binding affinity to colloidal Teflon and their structural determination using CD

A. Normalized CD spectra for the peptides that may or may not bind to colloidal Teflon. Thick solid line, supernatant after removal of the Teflon-bound species by centrifugation. Thin solid line, peptides still bound to the Teflon beads suspended in water. Dotted line, water resuspended Teflon-bound species which was subsequently heated to 65°C in the presence of 0.1% Tween80 to release the peptides from the Teflon beads.

B. Linear mode MALDI-TOF analysis of the supernatant after removal of the Teflon-bound species by centrifugation.

C. Linear mode MALDI-TOF analysis of the peptides still bound to the Teflon beads suspended in water.
Probing of the various states of SC3 by Amide hydrogen/deuterium exchange

1. Performic acid oxidation of hydrophobin SC3 does not change the binding properties

PFA-SC3, with eight negative charges introduced into the protein, did not assemble in solution, and the CD spectrum was typical of an unfolded peptide in solution (thick solid line, Fig. 5A). Upon binding to colloidal Teflon, PFA-SC3 converted to an α-helical-like secondary structure with a spectrum similar to that of native SC3 (thin solid line and dotted line, Fig. 5A). Further treatment by heating to 65°C in the presence of detergent converted the secondary structure of Teflon-bound PFA-SC3 from the α-helical to the β-sheet state, similar to that of native SC3 (thin solid line and dotted line, Fig. 5B). Iodoacetic acid-modified SC3 (IAA-SC3) has been reported to undergo similar structural changes upon binding to a Teflon surface layer (de Vocht et al., 2000). It thus seems that opening of the disulfide bonds and introduction of eight negative charges to SC3 only affects its solubility and structure in a solution, but does not significantly affect the surface binding properties of the protein or the structural states it assumes when bound to a hydrophobic surface.

We also observed that 10mM of calcium chloride converted unstructured PFA-SC3 in solution into a protein with α-helical-like structure (thick and thin solid lines, Fig. 5C). Subsequent lowering of the pH to 2 enhanced the formation of α-helical-like structure (dotted line, Fig. 5C). Other bivalent cations, such as Mg^{2+} and Mn^{2+}, were able to induce similar structural changes, resulting in an α-helical state. Possibly, bivalent cations interact with the negatively charged sulfonic acid groups of cysteic acids, and the salt bridges formed direct the folding of the unstructured SC3 into a characteristic α-helical-like structure. Monovalent cations, even at 50mM concentrations, lacked such a capability of converting the protein structure. The main reason for using bivalent-cation refolded PFA-SC3 in the H/D exchange experiment was to make a comparison with Teflon-bound PFA-SC3, which was more difficult to digest by pepsin than Ca^{2+}-treated PFA-SC3 (see next section).

2. Pepsin digestion of the various conformational states of SC3 and PFA-SC3

When soluble-state SC3 was directly digested by pepsin for 30 min at 0°C, only the fragments corresponding to the predicted 2nd loop could be detected on LC/MS (Fig. 6A). The rest of the molecule might have resisted pepsin digestion due to the presence of four disulfide bridges, and/or might also be difficult to ionize in LC/MS (similar to soluble-state SC3 without
digestion). PFA-SC3, on the other hand, was easily digested and a much higher sequence coverage was obtained (Fig. 6B). The peptides generated were reproducibly detected by LC/ESI-MS, and, with a combination of computer-assisted molecular weight analysis and the MS/MS technique, most of the peptides could be assigned to PFA-SC3. The pepsin digestion was also efficient with Ca$^{2+}$-treated PFA-SC3 and an almost equivalent number of peptides were observed with high intensities in LC/MS. Pepsin digestion of Teflon-bound PFA-SC3 turned out to be more problematic, and fewer peptic peptides and lower peak intensities were observed than in the Teflon-free and Ca$^{2+}$-treated sample. The exceptions were peptides L101-L112 and I102-L112, which correspond to the C-terminal end of SC3. Their abundance largely increased when the protein was converted from α-helical state into β-sheet state on Teflon. Some peptides were

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**Fig. 5** Conformational changes of intact PFA-SC3 upon binding to a Teflon surface or clustering with Ca$^{2+}$

A. CD spectra for PFA-SC3 and SC3 binding to colloidal Teflon. Thick solid line, PFA-SC3 in 10mM sodium phosphate, pH7. Thin solid line, PFA-SC3 binding to Teflon beads in 10mM sodium phosphate, pH7. Dotted line, SC3 binding to Teflon beads in 10mM sodium phosphate, pH7.

B. CD spectra for PFA-SC3 and SC3 binding to colloidal Teflon in the presence of hot detergent. Thin solid line, Teflon-bound PFA-SC3 that was subsequently treated by heating to 65 °C and adding Tween 80 to a final concentration of 0.1%. Dotted line, Teflon-bound SC3 that was subsequently treated by heating up to 65 °C and adding Tween 80 to a final concentration of 0.1%.

C. CD spectra for PFA-SC3 clustered with Ca$^{2+}$. Thick solid line, PFA-SC3 in water. Thin solid line, PFA-SC3 dissolved in water containing 10mM calcium chloride. Dotted line, PFA-SC3 dissolved in water containing 10mM calcium chloride and 0.1% TFA to lower the pH to approximately 2.
totally missing in the Teflon-bound state, especially those corresponding to the predicted 2nd loop. This could be due to poor pepsin digestion efficiency, which is most likely caused by the shielding of some pepsin cleavage sites when the protein is bound to a solid surface. Another explanation is that some peptic peptides bound to the Teflon surface so strongly that they were difficult to dissociate into the Teflon-free fraction. These data already give us a hint about the folding of Teflon-bound PFA-SC3 and supports the suggestion that the predicted 2nd loop strongly interacts with Teflon surface, whereas the C-terminal tail is exposed to the solvent, in particular when the protein is in the β-sheet state.

**Fig. 6** Pepsin digestion on SC3 and performic acid oxidized SC3 (PFA-SC3)
A. Amino acid sequence of SC3. Arrows indicate the peptic peptides generated by pepsin digestion.
B. Amino acid sequence of performic acid oxidized SC3 (PFA-SC3). Arrows indicate the peptic peptides generated by pepsin digestion. The asterisks indicate the oxidized cysteines upon PFA treatment.
3. *H/D exchange experiments reveal highly solvent protected and exposed regions in SC3*

Soluble-state SC3, Teflon-bound α-helical-state PFA-SC3, Teflon-bound β-sheet-state PFA-SC3 and Ca\(^{2+}\)-associated PFA-SC3 were exposed to D\(_2\)O at neutral pH, and the deuterium exchange was performed for periods ranging from 1 min to 48 h. In most cases, eleven time points were taken for each protein preparation, and the samples, together with 0% and 100% reference samples, were cooled on ice and quickly fragmented by pepsin at pH2. The pepsin fragmentation procedure was straightforward for soluble-state SC3 and calcium-associated PFA-SC3, but more complex for the Teflon-bound species, as illustrated in Fig. 7. The peptic peptides were analyzed by LC/MS and isotopic distributions were determined. Although MS signals were less intense for the Teflon-bound samples, isotopic distributions were still distinguishable in most cases. The exchange of deuterium into peptide amide bonds results in an increase of the molecular mass (Fig. 8 and Fig. 9). All the peptides detectable in the H/D exchange experiments showed a single envelope of isotope peaks, indicating that they were structurally homogeneous in the various states. In order to compare the deuterium contents of the different peptides, an
averaged mass was obtained by centroiding the isotopic peaks in terms of peak intensity and width.

![Graphs showing time-dependent deuterium exchange](image)

**Fig. 8** Time-dependent deuterium exchange as seen by the change of isotopic distributions for fragment L55-G59.
A. Deuterium in-exchange for soluble-state SC3.
B. Deuterium in-exchange for $\alpha$-helical-state PFA-SC3 on a Teflon surface.
C. Deuterium in-exchange for Ca$^{2+}$ associated $\alpha$-helical-state PFA-SC3.

The deuterium levels in a peptide were plotted against the exchange time, and the data were fitted with series of first-order exponential equations as indicated in Fig. 10 A and B for peptide N66-L68 and L55-G59, respectively. The kinetics of H/D exchange allows one to classify the exchangeable amide hydrogens in terms of rate constants (Table 2, 3, 4, 5). By comparing these H/D exchange rate constants, one can clearly see that soluble-state SC3, with only the 2nd loop and the C73-C86 segment being digested and observed, only showed fast or intermediate exchanging residues. The lack of highly H/D exchange protected regions might indicate that soluble-state SC3 has a relatively loose structure, at least in these two regions, and that the
residues were readily accessible from the solvent. On the other hand, highly H/D exchange protected regions could be found in the other three types of molecules, with a remarkable consistency in the first half of the predicted 2\textsuperscript{nd} loop (Fig. 11A). The peptic peptides in this region, A46-L54, L54-L57, L55-G59, and L58-L62, showed almost complete protection from deuterium labeling in the Teflon-bound forms. Some other peptic peptides also contained some slowly exchanging residues, however, due to the difficulty of assignment, they were not indicated in the figure. Once SC3 was bound to a Teflon surface and converted into the \(\alpha\)-helical state, 13 residues in the 2\textsuperscript{nd} loop were highly protected against deuterium exchange. The high protection might be caused by the formation of a core structure dominated by \(\alpha\)-helical elements.

![Fig. 9 Time-dependent deuterium exchange as seen by the change of isotopic distributions for fragment L101-L102.](image)

A. Deuterium in-exchange for \(\alpha\)-helical-state PFA-SC3 on a Teflon surface.
B. Deuterium in-exchange for \(\beta\)-sheet-state PFA-SC3 on Teflon.
C. Deuterium in-exchange for Ca\textsuperscript{2+} associated \(\alpha\)-helical-state PFA-SC3. Note that samples with shorter deuterium exchange time spans (from 1 min to 30 min) were indicated instead of longer time spans (from 5 min to 2 h) used in A and B.
that bind to the Teflon surface via its abundant hydrophobic residues. The highly protected region in the predicted 2nd loop of α-helical-state PFA-SC3 extended upon conversion into the β-sheet state, indicating that more residues in this region are involved in the structure formation. The α-helical-state structure induced by Ca$^{2+}$ has a smaller protected region in the 2nd loop compared to that in the Teflon associated α-helical state. Therefore, the structure of the 2nd loop might be similar for the Ca$^{2+}$- and Teflon-bound α-helical state, but with the latter being somewhat more structured. The peptide L101-L112, which corresponds to the last part of the 4th loop and the whole C-terminal tail, was detectable in all the states except for soluble state. In the α-helical state on Teflon, this peptide showed both intermediate and slow deuterium exchange (Table 3, Fig. 11A). Interestingly, the deuterium exchange rates for the same peptide increased dramatically upon the formation of β-sheet-state structure and Ca$^{2+}$ induced α-helical structure (Table 4, 5). This suggests that, in contrast to the α-helical state on Teflon, the C-terminal end of the molecule in the β-sheet state is largely exposed to the solvent. This suggestion is corroborated by the findings on the pepsin digestion efficiencies.

**Fig. 10** Exponential fitting according to equation 2 of time-dependent deuterium exchange data

A. N66-L68 of SC3 in four different conformational states.

B. L55-G59 of SC3 in four different conformational states.

Solid line indicates the fitting of the deuterium levels of a peptide generated from soluble state (■); dotted line, Teflon-bound α-helical state (●); dashed line, Teflon-bound β-sheet state (▲); dashed-dotted line, Ca$^{2+}$ associated α-helical state (▼).
Fig. 11 Structural information on various SC3 states related to the assignment of slowly deuterium exchanging residues in the second loop and C-terminal peptide.
A. The highly H/D exchange-protected regions in the second loop and C-terminus are indicated as black circles (k < 0.1 min⁻¹).
B. CD spectra of the chemically synthesized peptide (S47-L62) which corresponds to the highly H/D exchange protected region in β-sheet-state PFA-SC3. Thick solid line, peptide solubilized in 10mM sodium phosphate, pH7. Thin solid line, peptide binding to Teflon beads in 10mM sodium phosphate, pH7. Dotted line, 5min vortex of the peptide that was solubilized in 10mM sodium phosphate.

Chemical synthesis of a part of the predicted 2nd loop and its α-helical-state like structure on a Teflon surface

The 16 amino acid peptide, corresponding to the highly H/D exchange-protected region (S47-L62) in the predicted 2nd loop of SC3 molecule in Teflon-bound form (Fig. 11A), was synthesized. The peptide was very water insoluble, and, at the maximum soluble concentration, it yielded only a very low CD signal (thick solid line, Fig. 11B). However, once 10% (v/v) colloidal Teflon was added to the peptide solution, an α-helical-state like structure, with highly enhanced CD signal, could be observed (thin solid line, Fig. 11B). This α-helical-state-like structure was obviously caused by the peptide binding to the Teflon surface, because spinning down and subsequent resuspension of those Teflon beads in the same volume of buffer did not
change either the intensity or the shape of the spectrum (data not shown). Further studies also showed that hot detergent (e.g. 0.1% Tween 80, 65°C) could not reverse the binding of the peptide or alter the spectrum (data not shown). Furthermore, we also tested the possible structural changes in the peptide at the air/water interface, that is, under conditions where self-assembly/β-sheet-state structure formation of hydrophobin SC3 can be induced (de Vocht et al., 1998). As shown on Fig. 11B, after vortexing of the peptide solution for 5 minutes, a large structural change of the peptide was observed, which was accompanied with a large increase in CD signal (compare thick solid and dotted line). However, the spectrum is quite different from a typical CD spectrum of β-sheet-state SC3, which has a maximal ellipcity at about 215nm. These results suggests that this part of SC3 might be at least partially responsible for the α-helical-state structure of SC3 on a hydrophobic surface, and also contribute to the β-sheet-state structure formation of the whole molecule either on a hydrophobic surface or at an air/water interface.

Table 2. Distribution of rate constants for H/D exchange at amide bonds in peptic peptides generated from soluble-state SC3

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<tr>
<td>L54-L57</td>
<td>LLGL</td>
<td>415.4</td>
<td>3</td>
<td>1.1</td>
<td>1.2</td>
<td>0.7</td>
</tr>
<tr>
<td>L55-G59</td>
<td>LGLLG</td>
<td>472</td>
<td>4</td>
<td>1.6</td>
<td>1.7</td>
<td>0.7</td>
</tr>
<tr>
<td>L58-L62</td>
<td>LGIVL</td>
<td>514.3</td>
<td>4</td>
<td>2.6</td>
<td>1.3</td>
<td>0.1</td>
</tr>
<tr>
<td>I60-L62</td>
<td>IVL</td>
<td>344.2</td>
<td>2</td>
<td>1.1</td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
<td>I60-L65</td>
<td>IVLSDL</td>
<td>659.4</td>
<td>5</td>
<td>4.6</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>S63-V67</td>
<td>SDLNV</td>
<td>547.5</td>
<td>4</td>
<td>2.8</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>N66-G70</td>
<td>NVLVG</td>
<td>501.3</td>
<td>4</td>
<td>1.1</td>
<td>2.1</td>
<td>0.8</td>
</tr>
<tr>
<td>N66-L68</td>
<td>NVL</td>
<td>345.2</td>
<td>2</td>
<td>0</td>
<td>1.8</td>
<td>0.2</td>
</tr>
<tr>
<td>L76-S84</td>
<td>LTVIGVGGS</td>
<td>802.6</td>
<td>8</td>
<td>7.4</td>
<td>0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The number of peptide amide hydrogens that undergo fast, slow, very slow deuterium exchange were obtained with equation 2 and are described by N1, N2 and N3, respectively. NHs indicates the number of possible exchanged amide hydrogens; k indicates the rate constant (min⁻¹) obtained from equation 2.
In the present study, we used both MALDI-TOF and Electrospray mass spectrometry to identify the portions of hydrophobin SC3 that undergo conformational changes upon assembly. Characteristic conformational changes were found to accompany the self-assembly process. Based on the primary sequence, and by comparison with another class I hydrophobin COH1 from *Coprinus cinereus*, it was assumed that the segment T52-S63 of SC3, which is a part of the predicted 2nd loop, might form an amphipathic $\alpha$-helix with hydrophobic residues on one face and hydrophilic or small residues on the opposite face of the helix. This amphipathic $\alpha$-helix may form an anchor that binds the protein strongly to hydrophobic surfaces.

The use of a Teflon target and colloidal Teflon enabled us to successfully fish out the hydrophobic surface-binding-peptides from the Asp-N digestion mixture, which were assigned as the two halves of the predicted 2nd loop. The first half of the 2nd loop, residues C40-S63,
showed a relatively high MS signal on a Teflon target after washing. This might indicate that the first half played an important role in surface binding and α-helical structure formation, which is consistent with the observation that the Asp-N cleaved C40-S63 contains the amphipathic segment T52-S63.

Table 4. Distribution of rate constants for H/D exchange at amide bonds in peptic peptides generated from Teflon-associate β-sheet-state PFA-SC3

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Mass (Da)</th>
<th>Number of amidehydrogens&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NH&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>C&lt;sup&gt;39&lt;/sup&gt;-Q44</td>
<td>C&lt;sup&gt;*&lt;/sup&gt;C&lt;sup&gt;39&lt;/sup&gt;NQVQ</td>
<td>789.5</td>
<td>5</td>
</tr>
<tr>
<td>A46-L54</td>
<td>ASSSPVTAL</td>
<td>832.5</td>
<td>7</td>
</tr>
<tr>
<td>P50-L54</td>
<td>PVTAL</td>
<td>500.3</td>
<td>3</td>
</tr>
<tr>
<td>L54-L57</td>
<td>LLGL</td>
<td>415.4</td>
<td>3</td>
</tr>
<tr>
<td>L55-G59</td>
<td>LGLLG</td>
<td>472</td>
<td>4</td>
</tr>
<tr>
<td>L58-L62</td>
<td>LGIVL</td>
<td>514.3</td>
<td>4</td>
</tr>
<tr>
<td>L60-L62</td>
<td>IVL</td>
<td>344.2</td>
<td>2</td>
</tr>
<tr>
<td>V61-N66/L62-V67</td>
<td>VLSDLNLSDLNV</td>
<td>660</td>
<td>5</td>
</tr>
<tr>
<td>L65-S74</td>
<td>LNVLVGISC&lt;sup&gt;*&lt;/sup&gt;S</td>
<td>1051.7</td>
<td>9</td>
</tr>
<tr>
<td>N66-L68</td>
<td>NVL</td>
<td>345.2</td>
<td>2</td>
</tr>
<tr>
<td>V69-T77</td>
<td>VGISC&lt;sup&gt;*&lt;/sup&gt;SPLT</td>
<td>924.5</td>
<td>7</td>
</tr>
<tr>
<td>I71-V78</td>
<td>ISC&lt;sup&gt;*&lt;/sup&gt;SPLTV</td>
<td>867.5</td>
<td>6</td>
</tr>
<tr>
<td>L101-L112</td>
<td>LINIGC&lt;sup&gt;*&lt;/sup&gt;TPINIL</td>
<td>1331.8</td>
<td>10</td>
</tr>
<tr>
<td>I102-L112</td>
<td>INIGC&lt;sup&gt;*&lt;/sup&gt;TPINIL</td>
<td>1218.8</td>
<td>9</td>
</tr>
</tbody>
</table>

The asterisks indicate the oxidized cysteines upon PFA treatment.

<sup>a</sup> see table 2

Peptides responsible for the tight binding of β-sheet-state SC3 to Teflon could not be identified with the Teflon target procedure. The procedure of heating and treating with detergent caused the release of peptides from the Teflon, in contrast to the situation with intact SC3 which yielded tightly bound β-sheet rodlet structures. This apparent discrepancy could be explained by the molecular dynamic simulation of SC3 assembled at water/hexane interface, in which a long-range β-sheet network through the whole molecule was observed rather than a single structural element. The α-helical structure and surface binding affinity of Teflon-bound segments C40-
S63/D64-S72 were obviously disturbed by the heating and detergent-treatment, causing release from the surface rather than formation of the long-range β-sheet network. We have previously shown that conversion of SC3 from the α-helix to β-sheet structure on Teflon is accompanied by a major increase in protein-protein interaction as reflected in increased fluorescence resonance energy transfer. This suggests a movement of the molecules towards one another due to the heat/detergent treatment. While there are obviously enough interactions between the individual SC3 molecules and the Teflon surface to maintain or restore the surface-bound nature of SC3 during these association processes, this is not the case with the isolated second loop peptide.

Table 5. Distribution of rate constants for H/D exchange at amide bonds in peptic peptides generated from Ca²⁺-associated α-helical-state PFA-SC3

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Mass (Da)</th>
<th>Number of amide hydrogens&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NH&lt;s&gt;ᵦ&lt;/s&gt;</th>
<th>k&gt;1000</th>
<th>100&gt;k&gt;1</th>
<th>0.1&gt;k</th>
</tr>
</thead>
<tbody>
<tr>
<td>C´39-Q44</td>
<td>C´C´NQVQ</td>
<td>789.5</td>
<td>5</td>
<td>1.1</td>
<td>2.1</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>S47-L54</td>
<td>SSSPVTAL</td>
<td>761.36</td>
<td>6</td>
<td>1.5</td>
<td>0</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>L54-L57</td>
<td>LLGL</td>
<td>415.4</td>
<td>3</td>
<td>1.5</td>
<td>0.9</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>L55-G59</td>
<td>LGLLG</td>
<td>472</td>
<td>4</td>
<td>1.7</td>
<td>1.5</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>L58-L62</td>
<td>LGIVL</td>
<td>514.3</td>
<td>4</td>
<td>0.8</td>
<td>2.0</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>I60-L62</td>
<td>IVL</td>
<td>344.2</td>
<td>2</td>
<td>0.4</td>
<td>1.0</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>I60-L65</td>
<td>IVLSDL</td>
<td>659.4</td>
<td>5</td>
<td>1.1</td>
<td>2.7</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>S63-V67</td>
<td>SDLNV</td>
<td>547.5</td>
<td>4</td>
<td>1.9</td>
<td>1.8</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>N66-G70</td>
<td>NVLVG</td>
<td>501.3</td>
<td>4</td>
<td>1.6</td>
<td>2.2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>N66-L68</td>
<td>NVL</td>
<td>345.2</td>
<td>2</td>
<td>0.6</td>
<td>1.2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>V69-V78</td>
<td>VGISC´SPLTV</td>
<td>1023.5</td>
<td>8</td>
<td>4.5</td>
<td>3.2</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>I71-V78</td>
<td>ISC´SPLTV</td>
<td>867.5</td>
<td>6</td>
<td>4.4</td>
<td>1.4</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>I79-Q89</td>
<td>IGVGGSGC´SAQ</td>
<td>983.9</td>
<td>10</td>
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</tr>
<tr>
<td>L101-L112</td>
<td>LINIGC´TPINIL</td>
<td>1331.8</td>
<td>10</td>
<td>4.1</td>
<td>5.3</td>
<td>0.6</td>
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</tr>
<tr>
<td>I102-L112</td>
<td>INIGC´TPINIL</td>
<td>1218.8</td>
<td>9</td>
<td>3.3</td>
<td>5.3</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

The asterisks indicate the oxidized cysteines upon PFA treatment.

<sup>a</sup> see table 2.
H/D exchange in combination with LC/ESI-MS analysis was used to obtain more detailed structural information. Since pepsin cleavage is relatively unspecific, the bottleneck here was the assignment of overlapping peptic peptides. The mass increase upon oxidation of cysteine residues made the assignment even more difficult, and peptide sequencing using MSMS was necessary to unequivocally identify the peptides. For soluble-state SC3, only the predicted 2\textsuperscript{nd} loop and the segment following it could be identified after pepsin digestion, whereas the entire molecule of soluble-state PFA-SC3 could be cleaved as inferred from SDS-PAGE analysis. Either poor ionization in MS or resistance to proteolysis due to the folded structure or glycosylation prevented the identification of the N-terminal segment in both SC3 and PFA-SC3. The fact that SC3 and PFA-SC3 underwent similar structural changes upon binding to a hydrophobic surface, and the observation that bound PFA-SC3 was pepsin cleavable, made PFA-SC3 a good “analogue” of SC3 for further analysis.

The isotope patterns in the mass spectra of the peptic peptides reflects the distribution of deuterium among the peptides in the sample. A single envelope of isotope peaks is expected if the sample is structurally homogeneous and if the exchange is uncorrelated. Bimodal isotope patterns are expected otherwise (Smith et al., 1997; Raza et al., 2000). All the deuterium exchanged peptic peptides of SC3 showed a single envelope of isotope peaks, implying that the molecules in each state were homogenous in structure.

Back-exchange from deuterium to hydrogen can take place during the sample manipulation, since the reaction is highly dependent of temperature and pH (Zhang et al., 1993; Smith et al., 1997). Low temperature (4°C) and low pH (about 2) were used during pepsin digestion and LC/MS analysis, and proper corrections for back-exchange were made with 0% and 100% deuterated samples (see materials and methods). Under our experimental conditions, the back exchange could be kept below 20%, as determined for unstructured PFA-SC3. We should emphasize that due to relatively low peak intensities, the error in the average masses of the peptides derived from Teflon-bound samples and the error in the relevant exchange rates are larger than for the Teflon-free samples. For the data analysis, the exchange data could be fitted with three first-order exponentials in most cases.

Although the assignment of overlapping peptic peptides turned out to be difficult in our case, there is no doubt that most residues in the first half of the predicted 2\textsuperscript{nd} loop underwent slow H/D exchange once the molecule gained structures on a hydrophobic surface (Fig. 11A). Slowly exchanging residues were not found in the soluble state SC3 peptides which could be detected, indicating that at least these parts of the molecule lacked a tight core structure. If the number of
protected residues is relevant to the content of secondary structure elements, the change in protein structure, from disordered, ordered, to highly ordered (mature assemblage), should be observable in going from the soluble to α-helical and to β-sheet state. Indeed, such a sequence of events could be clearly observed for the predicted 2nd loop of SC3. The H/D exchange data also shows that the C-terminal tail of SC3 is probably exposed to the solvent when SC3 assembles on a hydrophobic surface and forms a β-sheet-state structure. This offers the possibility of engineering this part of the molecule in order to display fusion proteins and epitopes on a solid surface.

**Conclusion**

The structural changes in PFA-SC3 upon surface binding have been elucidated with a spatial resolution in the range of 3 to 12 amino acids. Although, in principle high-resolution structural information at residue-specific level can be obtained by NMR spectroscopy, H/D exchange in combination with LC/MS provided us with an easy and fast method to determine the gross organization of hydrophobin SC3.

**Materials and Methods**

**Materials**

Endoproteinase Asp-N was purchased from Roche Applied Science; pepsin was purchased from Boehringer Mannheim; immobilized pepsin (on cross-linked 6% agarose, 2-3 mg of pepsin/ml of gel) was purchased from Pierce; alpha-cyano-4-hydroxycinnamic acid (97%) and deuterium oxide (99.9 atom %D) were obtained from Aldrich; hydrogen peroxide (30%) and trifluoroacetic acid (TFA) were obtained from Merck. All other chemicals and reagents used were of the highest grade commercially available.

**Performic acid oxidation of SC3**

Performic acid was prepared by mixing 1 ml of 30% hydrogen peroxide with 9 ml of formic acid. The mixture was allowed to stand at room temperature for 2 h and then quickly chilled to 0°C in an ice bath before use. Oxidation was performed by adding 0.5 ml chilled performic acid into 1 mg freeze-dried SC3, and the solution was allowed to stand for 2 h in dark in an ice bath at 0°C. After reaction, the oxidized protein was separated from performic acid by loading the
reaction mixture onto a water equilibrated PD-10 desalting column; the column was eluted with water, and the eluant containing PFA-SC3 was collected. The isolated PFA-SC3 was then lyophilized and stored at room temperature until used.

**Circular Dichroism Spectroscopy (CD)**

Soluble-state SC3 was prepared by trifluoroacetic acid (TFA) treatment of lyophilized SC3 and subsequent dissolution of the TFA-free material in a buffer as described previously (Wang et al., 2002). Structured PFA-SC3 on a hydrophobic surface (Teflon) was prepared as described for the native SC3 (Wang et al., 2002). To obtain the α-helical-state structure, lyophilized PFA-SC3 was dissolved in water, normally in a concentration of about 100 μg/ml, followed by addition of colloidal Teflon until a surface coverage of the Teflon spheres of about 10% was achieved, based on the fact that 1.5 mg of SC3 can fully cover 1 m² of Teflon surface (de Vocht et al., 1998). The Teflon spheres were centrifuged at 8,000g for 10 min, and then resuspended in 10 mM ammonium phosphate (pH 6.8). To obtain β-sheet-state structure, the solution containing Teflon-bound α-helical-state SC3 was heated to 65°C followed by the addition of Tween 80 to a final concentration of 0.1%. After 30 min of incubation at 65°C, the Teflon spheres were centrifuged and then resuspended in 0.1% 10 mM ammonium phosphate (pH 6.8). Ca²⁺-induced structured PFA-SC3 was obtained by adding a 10 mM (final concentration) calcium chloride to PFA-SC3 dissolved in water.

CD spectra of SC3 were recorded from 190 nm to 250 nm on an Aviv 62A DS CD spectrometer, using a 1-mm quartz cuvette. The temperature was kept at 4°C and the sample compartment was continuously flushed with nitrogen gas. The final spectra were obtained by averaging 5 scans, using a bandwidth of 1 nm, a stepwidth of 1 nm, and a 5-s averaging per point. The spectra were then corrected for the background signal using a reference solution without the protein.

**Asp-N digestion on PFA-SC3 and Teflon binding experiments**

1. **Asp-N digestion and MALDI-TOF analysis**

   Approximately 2 μg of Asp-N was dissolved in 30 μl of distilled water, and then gently mixed with 500 μl PFA-SC3 (2 mg/ml) dissolved in 50 mM sodium phosphate, pH 7.5. The digestion was allowed to continue for 18 h at 37°C, and then stopped by freezing the solution at -20°C. When the digestion mixture was desalted, the ZipTipC18 (Millipore) procedure according to the
recommendation of the manufacturer was used. The purified samples in 50% acetonitrile/0.1% TFA were then either subjected to MALDI-TOF analysis or frozen at -20°C.

For the peptide determination, 10 μl of digestion mixture with or without ZipTipC18 desalting was mixed with an equal volume of 10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% TFA (v/v) in an eppendorf tube. Usually, the samples were diluted 5-fold to achieve MS spectra with the highest quality. Aliquots of 1.5 μl were applied on to the stainless-steel target and allowed to air dry. MALDI-TOF mass spectra were recorded with a Micromass Tofspec E MALDI time-of-flight mass spectrometer operated in either linear or reflectron mode. Spectra were calibrated externally.

2. Binding to a Teflon target

The Teflon target was prepared as follows. Teflon tape (Aldrich) was cut into 2mm × 30mm squares, which were flattened onto the stainless-steel target and fixed in position with tape at each end. The digestion mixture was mixed with the MALDI matrix and applied onto the Teflon target as described above. For the binding experiments, 2 μl of digestion mixture was loaded onto the Teflon target, and allowed to stand at room temperature for a few minutes before being removed. After being air-dried, the sample spot was washed 3 times by applying 2 μl of distilled water each time, which were then allowed to air dry. In a control sample, the 2 μl of digestion mixture was allowed to dry on the Teflon target without washing. Finally, 1.5 μl of MALDI matrix was applied to the dried spots and allowed to dry. MALDI-TOF analysis was then performed in the linear mode as described above.

3. Binding to colloidal Teflon

250 μl of Asp-N digestion mixture in 50 mM sodium phosphate, pH 7.5 was mixed with 150 μl of colloid Teflon (about 20% (w/v)), which yields approximately 10% surface coverage. Teflon spheres were then centrifuged, and the pellet was resuspended in 350 μl distilled water. CD measurements were done on the supernatant and the resuspended Teflon spheres, followed by lyophilizing the two species. Before MALDI-TOF analysis, the lyophilized samples were treated with TFA (in the case of resuspended Teflon spheres, TFA was removed to another tube after extraction), dried with a flow of nitrogen gas, and then dissolved in 10 μl of distilled water. MALDI-TOF analysis was performed on a stainless-steel target as described above.
**H/D exchange experiments**

1. *Pepsin digestion and identification of peptic fragments of PFA-SC3*

Pepsin digestion was carried out at an enzyme to substrate ratio of 1:1. Before use, 0.5ml immobilized pepsin slurry was washed twice with 1ml of chilled 0.1% TFA (pH of about 2) by vortexing and subsequently centrifuging for 2 min at 7,000g, and then the preparation was stored on ice. 0.5 mg lyophilized PFA-SC3 was dissolved in 0.5 ml chilled 0.1% TFA, and then added to the immobilized pepsin. Digestion was carried out for 6 min on ice with occasional mixing. The immobilized pepsin was removed by centrifugation for 1 min at 14,000g at 4°C, and the supernatant was transferred to an empty tube. The samples were stored at –20°C until further analysis.

The peptic peptides were detected by LC/MS, and then assigned on the basis of computer-assisted analysis of the molecular weights and the MS/MS technique. For each LC/MS experiment, 20 μl sample was analyzed on a LC-ESI triple quadrupole mass spectrometer (PE-Sciex API3000) using a C18 column (Xterra) at 200 μl/min flow rate and a gradient elution involving 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B. After washing with 10%B for 5 min, the peptides were eluted by going from 10 to 40% B in 35min, followed by 40 to 95% B in 10min. To obtain the optimal peptide signal, the effluent from the column was split before being introduced into the mass spectrometer. The eluted peptides showing significant intensities were then selected and analyzed using MS/MS, and the sequence information was then obtained based on the product ions generated.

2. *H/D exchange experiment on SC3/PFA-SC3 in different conformational states*

For soluble-state SC3, H/D exchange was initiated by diluting 10 mg/ml of soluble-state SC3 solution 20-fold with 50 mM ammonium phosphate/D2O, pD 7.5. The sample was then incubated at room temperature, and at each time point, 50 μl of sample was removed from the labeling solution and transferred to another tube chilled on ice, and 4 μl of chilled 5% TFA/D2O was added to quench the deuterium in-exchange (final pH was about 2), followed by the addition of immobilized pepsin (100μl slurry, 2 times washing with chilled 0.1%TFA). Digestion was carried out for 6 min on ice, and the immobilized pepsin was removed by centrifugation for 1 min at 14,000g at 4°C. The supernatant was then transferred to an empty tube and stored in liquid nitrogen until further analysis. Undeuteriated protein (0% reference) was prepared by
diluting the SC3 or PFA-SC3 solution 20-fold with 50 mM ammonium phosphate/H₂O, pH 7.5. The completely deuterated protein (100% reference) was prepared from PFA-SC3 in stead of SC3.

H/D exchange for Ca²⁺-associated PFA-SC3 was similar as described for soluble-state SC3, except that PFA-SC3 was dissolved in 10 mM CaCl₂/0.1% TFA and then diluted 20-fold with 10 mM CaCl₂/0.1% TFA/D₂O (pD about 2).

For Teflon-bound α-helical-state PFA-SC3, 0.5 ml of 10 mg/ml PFA-SC3 in 50 mM sodium phosphate, pH 6.8 was mixed with 250 μl freshly prepared colloidal Teflon and the mixture was allowed to stand at room temperature for 10 min before being subjected to centrifugation at 7,000g for 10 min. The pellet was then quickly suspended in 600 μl of 50 mM ammonium phosphate/D₂O, pD 7.5, and then incubated at room temperature with occasional mixing. At each time point, 50 μl of sample was removed from the labeling solution to another empty tube chilled on ice, and 4 μl of chilled 5% TFA/D₂O was added to quench the deuterium in-exchange (about pD 2), followed by the addition of 10 μl of 5 mg/ml pepsin solution in chilled 0.1% TFA. The rest of the procedure was the same as described for soluble state.

For β-sheet-state PFA-SC3 on Teflon spheres, the mixture of PFA-SC3 and colloidal Teflon was incubated at 65°C for 30 min in the presence of 0.1% Tween80 before the centrifugation. The rest of the procedure was identical as described for α-helical-state SC3.

3. LC/ESI-MS analysis

The LC/ESI-MS system with a faster HPLC elution profile was used to determine the deuterium content in a peptide. The setup was identical to that in the MS/MS analysis as described above. The 50 μl sample frozen in liquid nitrogen was quickly thawed at 30°C and loaded to a 20 μl injection loop, followed by starting the HPLC elution program. After desalting at 10% B for 3 min, the protein was eluted by going from 10 to 40% B in 5 min, followed by 40 to 95% B in 2 min. Each run of HPLC needed less than 30 min. The HPLC injector and the column were submerged in ice/water bath, and the transfer syringe was rinsed with D₂O and precooled on ice before use.

4. Data analysis

To obtain an average molecular weight of a peptide, the data were processed by centroiding an isotopic distribution corresponding to the +1 charge state of each peptide. Corrections were made
for deuterium back-exchange during analysis as reported (Zhang et al., 1993). With each set of samples, an undeuterated (0% reference) control and a completely deuterated (100% reference) control were also analyzed. Equation 1 was used to apply the correction, where \( D \) is the deuterium content of the peptide, and \( m, m_{0\%}, \) and \( m_{100\%} \) are the average molecular weights of the same peptide in the partially deuterated, the undeuterated, and the completely deuterated form, respectively, and \( N \) is the number of peptide amide hydrogens.

\[
D = \frac{m - m_{0\%}}{m_{100\%} - m_{0\%}} \times N \quad (1)
\]

Deuterium levels (\( D \)) were then plotted versus the exchange time (\( t \)) and fitted with a series of first-order rate expressions according to equation 2, where \( N \) is the same as in equation 1, and \( k_i \) is the hydrogen deuterium-exchange rate constant for each peptide linkage.

\[
D = N_1[1-\exp(-k_1t)] + N_2[1-\exp(-k_2t)] + N_3[1-\exp(-k_3t)] \quad (2)
\]

**Peptide synthesis**

A 16-amino acid peptide (Ac-Ser-Ser-Ser-Pro-Val-Thr-Ala-Leu-Leu-Gly-Leu-Leu-Gly-Ile-Val-Leu-NH\(_2\)), which corresponds to the highly deuterium protected region in \( \alpha \)-helical-state SC3 was synthesized as follows. Individual N-ace tyl peptide-amide derivatives were assembled on an automatic ABI 433A Peptide Synthesize r using the ABI FastMoc 0.25 mmol protocols (Fields et al., 1991) except that the coupling time was 45 min instead of 20 min. Fmoc-amino acid derivatives, activated in situ using HBTU/HOBt and DiPEA in NMP, were used in the coupling steps. The peptides were deprotected and cleaved from the resin by treatment with TFA/TIS/H\(_2\)O (95/2.5/2.5, v/v/v) at room temperature for 2 h. After this, the peptides were precipitated by MTBE/n-hexane (1/1, v/v) solvent mixture. Finally, the pellet was dissolved in MeCN/H\(_2\)O (1/1, v/v) and lyophilized to obtain the crude peptides. The crude peptides were purified by preparative C8 or C18 reversed-phase HPLC. The identity of the peptide was confirmed by MALDI-TOF MS. Similar to the treatment of SC3, the lyophilized peptide was first treated with pure TFA and dried with a flow of nitrogen gas. The dried material was then dissolved in water before being analyzed.